



# Mitochondrial respiratory chain involvement in peroxiredoxin 3 oxidation by phenethyl isothiocyanate and auranofin

Kristin K. Brown<sup>1</sup>, Andrew G. Cox<sup>1</sup>, Mark B. Hampton<sup>\*</sup>

Free Radical Research Group and National Research Centre for Growth and Development, Department of Pathology, University of Otago, Christchurch, New Zealand

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## ABSTRACT

**Mitochondrial peroxiredoxin 3 (Prx 3) is rapidly oxidized in cells exposed to phenethyl isothiocyanate (PEITC) and auranofin (AFN), but the mechanism of oxidation is unclear. Using HL-60 cells deplete of mitochondrial DNA we show that peroxiredoxin 3 oxidation and cytotoxicity requires a functional respiratory chain. Thioredoxin reductase (TrxR) could be inhibited by up to 90% by auranofin without direct oxidation of peroxiredoxin 3. However, inhibition of thioredoxin reductase promoted peroxiredoxin 3 oxidation and cytotoxicity in combination with phenethyl isothiocyanate or antimycin A. We conclude that rapid peroxiredoxin 3 oxidation occurs as a consequence of increased oxidant production from the mitochondrial respiratory chain.**

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## 1. Introduction

Peroxiredoxin 3 (Prx 3) is an abundant mitochondrial protein that reacts rapidly with hydrogen peroxide, and is likely to be a key regulator of mitochondrial hydrogen peroxide levels [1]. Prx 3 is oxidized by hydrogen peroxide to a disulfide-linked dimer, and is dependent on the thioredoxin system for recycling to the reduced form. Accumulation of oxidized Prx 3 has been detected during ischemia [2] and apoptosis [3–5] and in all of these models the redox status of the cytoplasmic Prxs are unaltered, indicating a specific mitochondrial event.

Prx 3 has been proposed to play a role in the regulation of apoptosis, with alterations in expression modulating cellular sensitivity to pro-apoptotic stimuli [6,7]. While various redox events are proposed to be involved in apoptosis signaling, it is difficult to determine whether they are a cause or consequence of apoptosis induction. Two inducers, phenethyl isothiocyanate (PEITC) and auranofin (AFN), trigger substantial oxidation of Prx 3 within 30 min and prior to other major apoptotic events [4,5], indicating

an early disruption of mitochondrial redox homeostasis. However, the mechanism underlying the oxidation of Prx 3 and its significance is unclear.

Prx 3 oxidation could result from increased mitochondrial hydrogen peroxide production or impairment of the thioredoxin (Trx)/thioredoxin reductase (TrxR)/NADPH system responsible for reducing Prxs. The major source of mitochondrial hydrogen peroxide is dismutation of superoxide generated by respiratory complexes [8]. In this study we compared the response of wild-type HL-60 leukemia cells with those lacking mitochondrial DNA to determine the importance of a functional respiratory chain for Prx 3 oxidation. AFN, an inhibitor of TrxR [9], was also used to determine the relative importance of thioredoxin system in maintaining Prx 3 in its reduced form.

## 2. Materials and methods

### 2.1. Materials

Cell culture materials were from Invitrogen New Zealand Ltd. (Auckland, New Zealand). PEITC was from Sigma Chemical Co. (St. Louis, MO, USA). Auranofin was from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Complete™ protease inhibitors and CHAPS were from Roche Diagnostics (Mannheim, Germany). Rabbit polyclonal antibodies to Prx 3 and Trx 1 were from Abfrontier Co.

Abbreviations: AFN, auranofin; NEM, N-ethylmaleimide; PEITC, phenethyl isothiocyanate; Prx, peroxiredoxin; p<sup>0</sup>, mitochondrial DNA deplete; TrxR, thioredoxin reductase

<sup>\*</sup> Corresponding author. Fax: +64 3 364 1083.

E-mail address: [mark.hampton@otago.ac.nz](mailto:mark.hampton@otago.ac.nz) (M.B. Hampton).

<sup>1</sup> These authors contributed equally to this work.

Ltd. (Seoul, Korea) and goat polyclonal antibody to Trx 2 was from R&D Systems (Minneapolis, MN, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were from Sigma Chemical Co. Hybond-PVDF membrane and enhanced chemiluminescence (ECL<sup>TM</sup>) western blotting system were from GE Healthcare (Buckinghamshire, England). All other chemicals and reagents were from Sigma Chemical Co. and BDH Laboratory Supplies (Poole, England).

## 2.2. Cell culture

The mitochondrial DNA deplete ( $\rho^0$ ) and WT HL-60 cells used in this study were generously provided by Prof. Mike Berridge of the Malaghan Institute of Medical Research (Wellington, New Zealand). The  $\rho^0$  cells had been generated by culturing HL-60 cells in the presence of ethidium bromide for 6–8 weeks and the lack of mtDNA confirmed by PCR [10]. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamate, 1 mM pyruvate and 50  $\mu$ g/ml uridine, and maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells were harvested in the exponential phase of growth (between 0.4 and 0.8  $\times 10^6$  cells/ml). WT and  $\rho^0$  HL-60 cells were cultured and treated in full DMEM media containing pyruvate and uridine. Removal of pyruvate and uridine halted growth of the  $\rho^0$  cells but had no effect on WT cell growth (not shown).

## 2.3. Immunoblot detection of the Prxs

Treated cells were pelleted and resuspended in N-ethylmaleimide (NEM) buffer (40 mM HEPES, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitors, and 100 mM NEM). After 15 min incubation cells were lysed by addition of 1% CHAPS and clarified by centrifugation. Lysates containing 10  $\mu$ g protein were combined in a 2:1 ratio with non-reducing sample loading buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue) and resolved by SDS–PAGE. Proteins were transferred to PVDF membrane, probed with primary and secondary antibody and visualized by using a peroxidase system with enhanced chemiluminescence (ECL<sup>TM</sup>). Images were obtained using a ChemiDoc<sup>TM</sup> XRS system (Bio-Rad, Hercules, CA) and densitometry of scanned images was undertaken using Quantity One<sup>®</sup> software (Bio-Rad, Hercules, CA).

## 2.4. Flow cytometric analysis of cell viability

Treated cells ( $2 \times 10^5$ ) were resuspended in PBS containing 5  $\mu$ g/ml PI. Cells were incubated in the dark for 10 min before analysis of the percentage PI-positive cells using a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Fullerton, CA).

## 2.5. Immunoblot detection of the redox state of the Trxs

Treated cells ( $2 \times 10^6$ ) were pelleted and resuspended in 80  $\mu$ l NEM buffer containing 1% CHAPS. After 30 min incubation lysates were clarified by centrifugation and desalted using a micro spin column (Bio-Rad, Hercules, CA, USA) pre-equilibrated with extract buffer (40 mM HEPES, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA). The eluant was incubated with 5 mM dithiothreitol for 30 min before alkylation with 25 mM  $\alpha$ -methoxy- $\omega$ -ethylmaleimide poly(ethylene glycol) 2 kDa (MalPEG-2kDa, Iris Biotech GmbH, Marktredwitz, Germany) for 30 min. Alkylated protein was extracted by acetone precipitation, resuspended in reducing sample buffer (sample loading buffer containing 700 mM  $\beta$ -mercaptoethanol), resolved by SDS–PAGE and detected by immunoblotting as described above.

## 2.6. Preparation of mitochondrial and cytosolic fractions

Following treatment,  $3 \times 10^7$  HL-60 cells were collected and resuspended in 1 ml mitochondrial isolation buffer (20 mM HEPES–KOH, pH 7.5, containing 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose and protease inhibitors). Selective permeabilization of the plasma membrane was achieved by addition of 100  $\mu$ g digitonin. Samples were briefly vortexed before centrifugation at 650 $\times$ g for 10 min. The supernatant was removed and centrifuged at 12 500 $\times$ g for 10 min. The resulting supernatant (cytosolic fraction) was removed while the pellet (mitochondrial fraction) was resuspended in 50  $\mu$ l of mitochondrial isolation buffer containing 2% CHAPS. Mitochondrial fractions were clarified by centrifugation at 15 000 $\times$ g for 4 min. The purity of the cytosolic and mitochondrial preparations was confirmed by immunoblotting against Prx 2 (cytosolic) and Prx 3 (mitochondrial) under reducing conditions.

## 2.7. TrxR assay

The activity of TrxR was measured using a modified DTNB reduction assay [11]. Treated cells were pelleted and resuspended in extract buffer (40 mM HEPES, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing 1% CHAPS and protease inhibitors. Cell lysates (or subcellular fractions) were transferred to a microplate and mixed with 200  $\mu$ l of 5 mM DTNB in PE buffer (100 mM potassium phosphate, pH 7.0, 10 mM EDTA) and the reaction was initiated by addition of 200  $\mu$ M NADPH. The relative activity of TrxR was determined as the difference between  $\Delta A_{412\text{ nm}}$  before and after the addition of NADPH.

## 2.8. Statistics

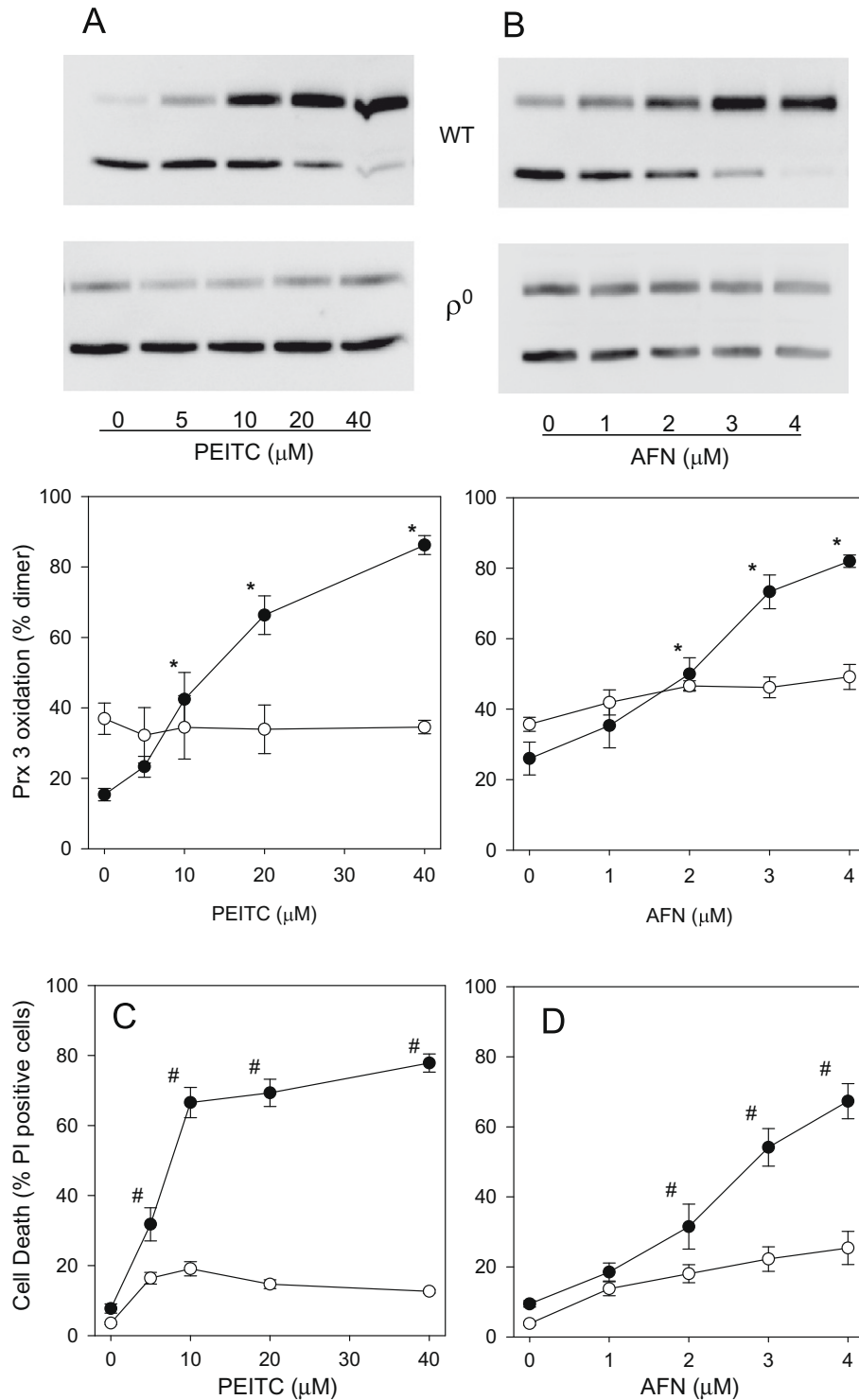
Statistical analyses were performed with the software package SigmaStat (Systat, San Rafael, CA, USA).

## 3. Results

Conversion of Prx 3 reduced monomer to oxidized dimer was observed following treatment of wild-type (WT) HL-60 cells with PEITC and the TrxR inhibitor AFN (Fig. 1A and B). To determine the role of mitochondrial respiratory complexes in the oxidation of Prx 3, we also assessed the response of  $\rho^0$  HL-60 cells. No increase in oxidation was observed following treatment of  $\rho^0$  HL-60 cells with PEITC (Fig. 1A) or AFN (Fig. 1B). Assessment of cell viability 24 h after treatment revealed that the  $\rho^0$  cells were also considerably more resistant than WT HL-60 cells to both compounds (Fig. 1C and D). This indicates that a fully functional respiratory chain is critical for both Prx 3 oxidation and subsequent cytotoxicity.

Given that the redox state of Prx 3 is regulated by Trx 2, we examined the redox status of Trx 1 (cytosolic) and Trx 2 (mitochondrial) following treatment with PEITC or AFN. The redox state of Trx was investigated by labeling reversibly oxidized thiols with MalPEG-2kDa and visualizing migration shifts by immunoblotting. Consistent with the Prx 3 results, Trx 2 was almost completely converted to an oxidized form in WT HL-60 cells following treatment with PEITC (Fig. 2A) or AFN (Fig. 2B), yet there was only a subtle increase in Trx 2 oxidation in the  $\rho^0$  HL-60 cells. In contrast, only minor Trx 1 oxidation was observed and this was similar in both WT and  $\rho^0$  HL-60 cells.

The ability of PEITC and AFN to inhibit TrxR was assessed in both WT and  $\rho^0$  HL-60 cells. PEITC inhibited TrxR in a dose-dependent manner with an IC<sub>50</sub> of 20  $\mu$ M, while AFN was considerably more potent with an IC<sub>50</sub> of 0.25  $\mu$ M (Fig. 3A). There was no

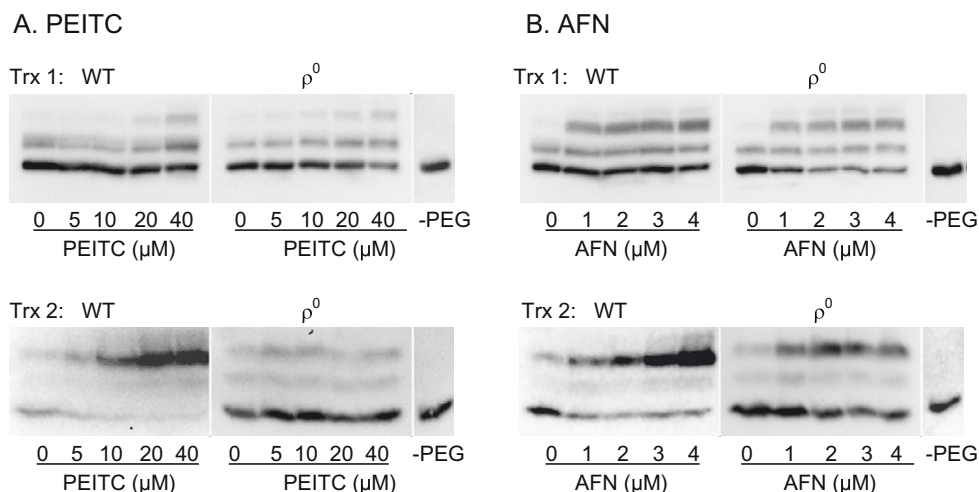


**Fig. 1.** PEITC and AFN induced Prx3 oxidation and cytotoxicity requires a functional respiratory chain. (A) and (B) WT (●) and  $\rho^0$  HL-60 (○) cells were exposed to 0–40  $\mu$ M PEITC (A) or 0–4  $\mu$ M AFN (B) for 60 or 30 min, respectively, before cells were harvested. Prx 3 oxidation was monitored by western blotting of non-reducing SDS–PAGE gels. Densitometry of scanned images using Quantity One software (Bio-Rad) was undertaken to quantify Prx 3 oxidation. \* Indicates a significant difference ( $p < 0.001$ ) between treated and untreated cells as determined by a one-way repeated measures ANOVA with Holm–Sidak multiple comparison (Systat). (C) and (D) Cell viability was measured after 24 h exposure under the same conditions as described above. Values are the mean and S.E. of four independent experiments. # Indicates a significant difference ( $p < 0.001$ ) between cell types at each dose as determined by a two-way repeated measures ANOVA with Bonferroni multiple comparison (Systat).

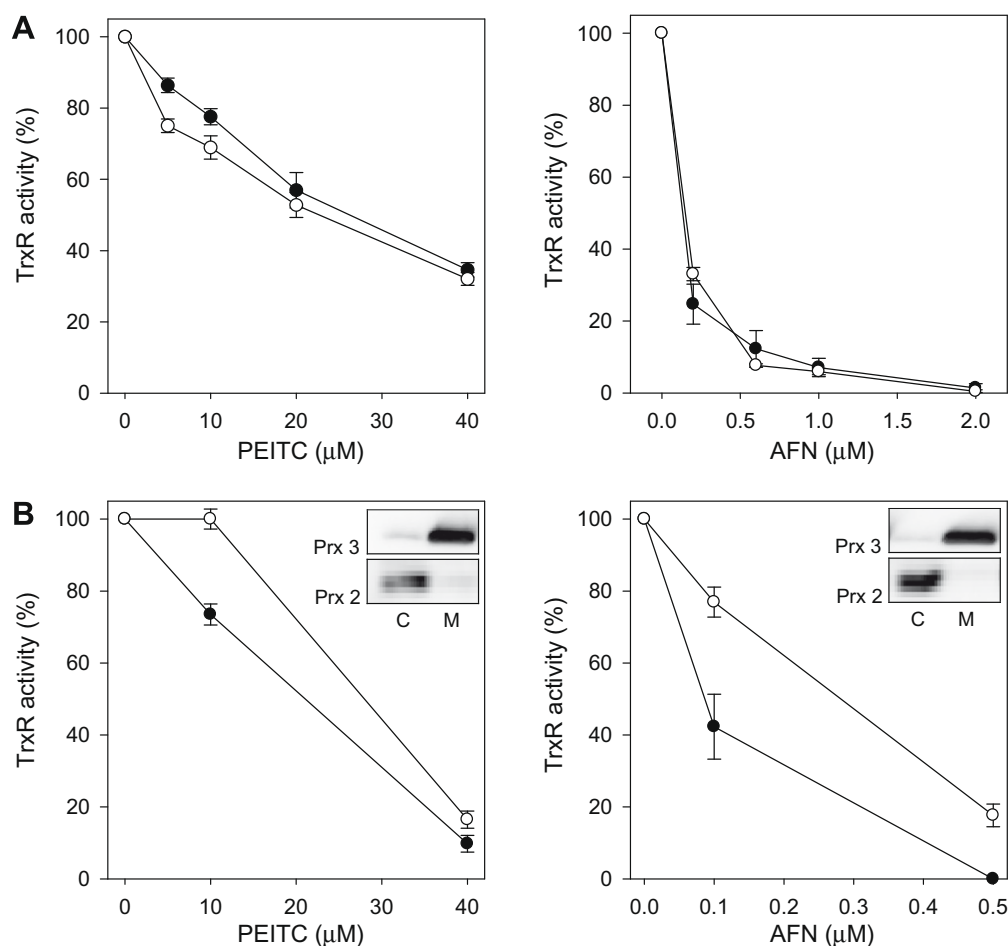
difference in inhibition between WT and  $\rho^0$  cells (Fig. 3A). Close inspection of the AFN dose response data reveals that AFN almost completely inhibited TrxR activity at doses that had little effect on the redox state of Prx3. For example, 0.5  $\mu$ M AFN inhibited TrxR by approximately 90%, but significant Prx 3 oxidation was not detected until cells were exposed to 2  $\mu$ M AFN (Figs. 3A and 1B).

Since PEITC had a limited effect on TrxR activity at concentrations that oxidized Prx 3 (Figs. 3A and 1A), we conclude that it is operating independent of its effect on TrxR.

To confirm that both compounds were able to effectively inhibit both TrxR1 (cytosolic) and TrxR2 (mitochondrial), HL-60 cells were fractionated into cytosolic and mitochondrial preparations prior to



**Fig. 2.** PEITC and AFN induced oxidation of Trx 2 requires a functional respiratory chain. WT and  $\rho^0$  HL-60 cells were exposed to 0–40  $\mu$ M PEITC (A) or 0–4  $\mu$ M AFN (B) for 60 or 30 min, respectively, before being harvested and resuspended in buffer containing NEM. Reversibly oxidized thiols were PEGylated and samples were immunoblotted for Trx 1 and Trx 2. Blots are representative of three independent experiments.



**Fig. 3.** PEITC and AFN inhibit TrxR. Cells were exposed to PEITC or AFN for 60 or 30 min, respectively, before being harvested for extraction. (A) PEITC and AFN inhibit TrxR activity in both WT (●) and  $\rho^0$  (○) HL-60 cells. Cell lysates were assessed for TrxR activity by measuring the NADPH-dependent reduction of DTNB. The specific TrxR activity in untreated WT and  $\rho^0$  HL-60 cells was similar (WT =  $273 \pm 26$   $\Delta$ mAU<sub>412nm</sub>/min/mg;  $\rho^0$  =  $263 \pm 34$   $\Delta$ mAU<sub>412nm</sub>/min/mg). (B) PEITC and AFN inhibit both cytosolic (●) and mitochondrial (○) TrxR activity. Cells were fractionated into cytosolic and mitochondrial extracts and TrxR activity in each fraction was measured. Inset: Purity of the cytosolic (C) and mitochondrial (M) extracts was confirmed by immunoblotting against Prx 2 (cytosolic) and Prx 3 (mitochondrial). Values are the mean and S.E. of three independent experiments.

the determination of TrxR activity. Consistent with our previous studies [4,5], PEITC and AFN inhibited both isoforms of TrxR, with cytosolic TrxR1 exhibiting a slightly greater extent of inhibition

than mitochondrial TrxR2 (Fig. 3B). Consequently, the oxidation of Prx 3 observed with PEITC and AFN does not seem to be associated with differential inhibition of mitochondrial TrxR.

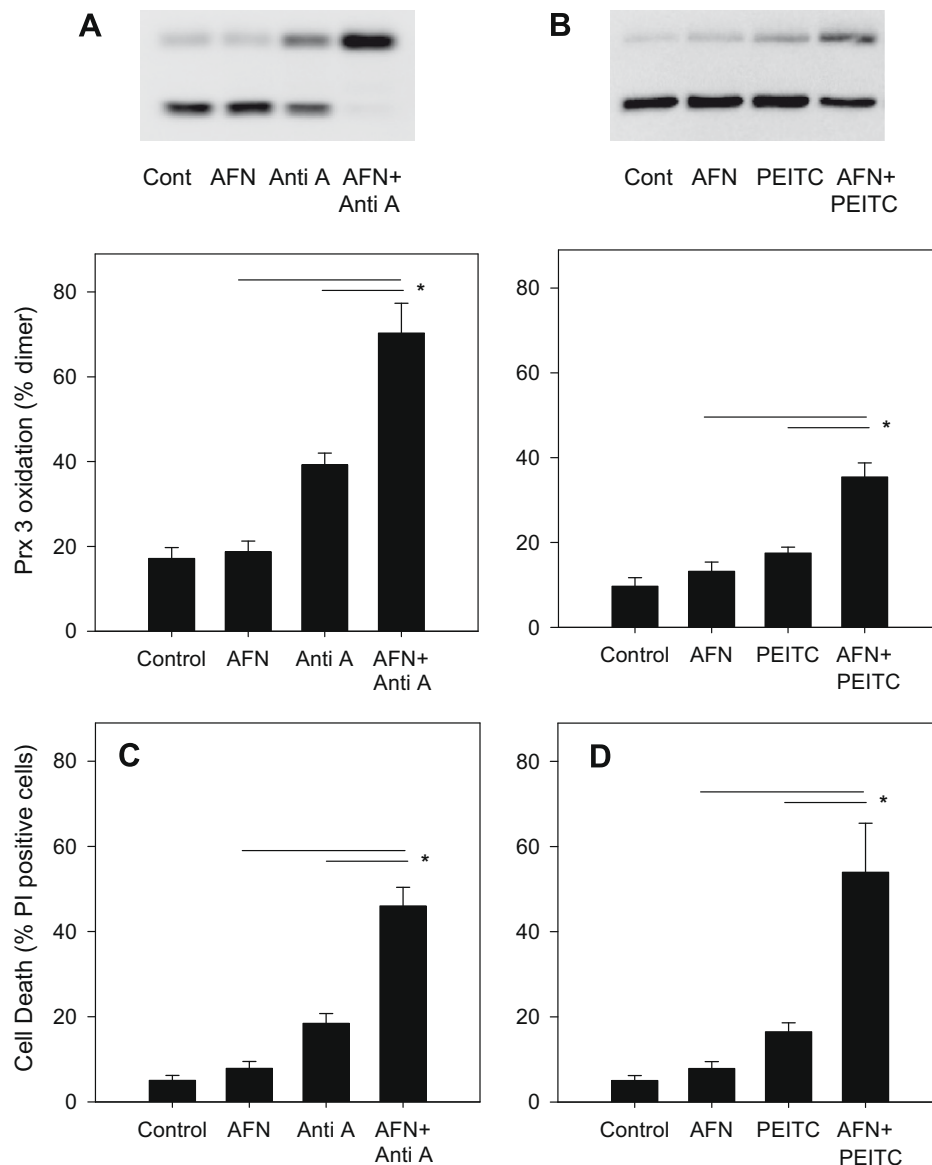
Low doses of AFN were used to determine if TrxR inhibition could sensitize Prx 3 to oxidation. The complex III inhibitor antimycin A increases mitochondrial oxidant production [12], and it triggered Prx 3 oxidation in HL-60 cells (Fig. 4A). Antimycin A did not affect cellular TrxR activity, and it did not cause Prx 3 oxidation in the  $p^0$  HL-60 cells (data not shown). AFN at 0.5  $\mu$ M promoted the oxidation of Prx 3 in the presence of antimycin A (Fig. 4A), with a synergistic increase in cytotoxicity (Fig. 4C). AFN was also able to sensitize cells to PEITC-triggered Prx 3 oxidation and cytotoxicity (Fig. 4B and D).

#### 4. Discussion

Oxidation of mitochondrial Prx 3 has been observed following treatment of cells with isothiocyanates and AFN [4,5], but the mechanism of oxidation has not been elucidated. Two possibilities exist for promoting the accumulation of oxidized Prx 3: an increase in mitochondrial oxidant production, or direct inhibition of the

Trx/TrxR/NADPH system necessary for recycling the oxidized protein. We have determined that the redox state of Prx 3 is resilient to inhibition of TrxR over the short periods examined in this study. While the TrxR inhibitor AFN caused Prx 3 oxidation at high doses, it was unable to trigger rapid accumulation of oxidized Prx 3 at doses where substantial inhibition of TrxR activity occurred. Instead, increased oxidation was dependent on a functional mitochondrial respiratory chain, indicating a central role for hydrogen peroxide generated by dismutation of superoxide derived from respiratory complexes. We therefore propose that PEITC increases the rate of oxidant generation from the respiratory chain. It is also possible that higher concentrations of AFN increase the endogenous flux of respiratory chain oxidants, but that is more difficult to separate from its effects on TrxR activity at lower doses. Regardless, the mitochondrial respiratory chain is vital for driving Prx 3 oxidation and the subsequent cytotoxicity associated with both compounds.

Mitochondria have been reported to be involved in isothiocyanate- and AFN-mediated cytotoxicity [5,13–15], primarily through



**Fig. 4.** TrxR inhibition sensitizes Prx 3 to oxidation by antimycin A and PEITC and enhances cytotoxicity. Prx 3 oxidation was measured in HL-60 cells following exposure to 10  $\mu$ M antimycin A for 30 min (A) or 5  $\mu$ M PEITC for 60 min (B) in the presence or absence of 0.5  $\mu$ M AFN. (C) and (D) Cell viability was measured after 24 h exposure under the same conditions as described above. Values are the mean and S.E. of at least three independent experiments. \* Indicates a significant difference ( $p < 0.05$ ) between indicated treatments as determined by a one-way repeated ANOVA with Holm-Sidak multiple comparisons (Systat).



the utilization of conventional mitochondrial-dependent apoptosis pathways. There is, however, evidence for a direct effect of isothiocyanates on mitochondrial function [16–18]. The exact mechanisms are not clear, with one group linking a small inhibitory effect on complex III to apoptosis [18], and the other proposing the effect is independent of the direct targeting of respiratory complexes [16]. Isothiocyanate-affinity probes may prove valuable in elucidating direct targets within mitochondria [19]. AFN has been shown to increase hydrogen peroxide production by isolated mitochondria, but not in submitochondrial particles with a functional respiratory chain, therefore it was concluded to primarily act through impairment of thioredoxin-dependent antioxidant defense [20].

Prx 3 appears to be a valuable marker of mitochondrial redox homeostasis. It is present endogenously at high concentrations, thereby eliminating the need for chemical targeting or expression, and it is extremely reactive [21,22]. It is difficult to determine how observations obtained with global redox-sensitive probes such as dichlorofluorescein relate to mitochondria [18,23], but the localization of Prx 3 ensures specificity. The disadvantage is that the oxidized Prx 3 is continually recycled by the thioredoxin system. However, the fortuitous observation reported here, that inhibition of TrxR by AFN alone does not immediately trigger Prx 3 oxidation, means that low dose AFN can be used to improve the sensitivity of Prx 3 oxidation as a biomarker.

There is also growing interest in the development of anti-cancer agents that act on mitochondria [24]. PEITC has been shown to effectively, and in some cases selectively, sensitize or kill drug-resistant and oncogenically-transformed cells [23,25–30]. AFN and related gold complexes are also being explored as anti-cancer agents [31–33]. From the synergistic effects of AFN observed on both Prx 3 oxidation and cytotoxicity, we speculate that TrxR inhibitors may function effectively in combination with other mitochondrial disrupting agents by overcoming the protection afforded by Prx 3.

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